In: Jankovic J, ed. •Neurological Disease and Therapy. Therapy with botulinum•toxin;1994;25:pp. 41-49
94050998

3

Preparation and Characterization of Botulinum Toxin Type A for Human Treatment

Edward J. Schantz and Eric A. Johnson
University of Wisconsin, Madison, Wisconsin

INTRODUCTION

The treatment of many hyperactive muscle disorders by injection of botulinum toxin (BTX) directly into a specific muscle has brought relief to thousands of people and has opened a new field of study on the application of the toxin to nerve and muscle tissue in the human body. During the past 23 years of developmental work on the use of the toxin for human treatment, selective procedures for the production, purification, and dispensing of the toxin have been developed to make it suitable for injection. In December 1989 the U.S. Food and Drug Administration (FDA) licensed botulinum toxin type A (BTX-A) (batch #79-11) as an orphan drug for the treatment of several dystonias and movement disorders.

The purpose of this paper is to describe (1) the essential points of production, purification and characterization of the type A toxin, (2) the specifications used to define the quality of the toxin that was produced at our laboratory at the University of Wisconsin for human treatment, and (3) the use of other serological types of toxin and their application to human treatment. It is important also to bring out developments during the past 23 or more years of research and experimentation that took place between Alan B. Scott and E. J. Schantz and later, in 1985, E.A. Johnson on type A toxin and its application to human treatment. We also propose improvements for attaining and maintaining the highest quality toxin for human treatment. The clinical work using type A toxin was first reported by Scott during the 1980s (1,2), and the properties of the toxin in relation to its use for human treatment were reported by Schantz and Scott in 1981 (3) and later by Schantz and Johnson in 1992 (4).

DEVELOPMENTS LEADING TO THE USE OF BOTULINUM TOXIN TYPE A

Type A is one of seven immunologically distinct types (A through G) of BTX composing a family of similar neurotoxins that produce paralysis by blockage of the release of acetylcholine at the myoneural junction. Because of the apparent high toxicity of type A toxin and its involvement in many outbreaks of food-borne botulism in the United States, it became the first to be investigated to any extent chemically and pharmacologically (3) and as a result was the first to be suggested for human treatment. The first reported attempt at purification of the type A toxin was carried out at the Hooper Foundation, University of California, San Francisco, in 1928 by Snipe and Sommer (5), who found that 90% or more of the toxin produced in deep culture broth could be precipitated by adjusting the pH of the spent culture to 3.5 with acid. This light brown, mud-like precipitate was the source of toxin for many pharmacological investigations and was even considered as a possible biological warfare agent against our troops during World War II. It was therefore investigated at Fort Detrick, Maryland, where it was isolated in crystalline form and many of its chemical, physical, and biological properties were determined (6,7). The Fort Detrick laboratory also furnished crystalline type A toxin to many research laboratories throughout the world that also contributed much to the knowledge of the structure and action of the toxin.

Although BTX had been studied for many years, investigations leading specifically to the use of BTX-A for the treatment of involuntary hyperactive muscle disorders came about through the collaborative work of Scott at Smith-Kettlewell Eye Research Institute in San Francisco and Schantz, who carried out research on several microbial toxins including BTXs from 1944 to 1971 at Fort Detrick, Maryland, and later from 1972 to the present at the Food Research Institute at the University of Wisconsin. The work was initiated by Dr. Scott, who had been searching for a substance to inactivate a hyperactive muscle in experimental rhesus monkeys in which he surgically produced a condition similar to strabismus. The object of his research was to find an alternative to surgery for human strabismus. Among several purified toxins Schantz had on hand, BTX-A was chosen for tests in Scott's monkeys because of its long paralyzing action in animals and in survivors of human cases of botulism. Several trials with the toxin brought about alignment of the eyes in the monkeys and encouraged further experimentation. Preparation of different batches of toxin was carried out to supply toxin for the monkey experiments, and improved results were obtained as Scott developed his technique for treatment. After about 10 years of experimentation, the U.S. Food and Drug Administration approved Scott's investigational new drug application for clinical tests on human volunteers.

For the human trials, particular attention had to be paid to the quality of the toxin (3). Although no protocols were available for the preparation of a protein drug of this type for injection, the use of the toxin for human experimentation clearly indicated that purity and toxicity (paralyzing activity) were important factors if FDA licensure was to be obtained. For these trials, a batch of about 200 mg of recrystallized toxin (batch 79-11) was prepared in November 1979 according to a standard procedure established by Schantz for toxin preparation and purification, with the following specifications on chemical, physical, and biological properties to define quality for this batch and all subsequent batches:

1. Production of toxin with the Hall strain of type A *Clostridium botulinum* in a simplified medium of 2% hydrolyzed casein, 1% yeast extract, and 0.5% dextrose (designated the production medium) containing no animal meat products

2. Purification by repeated precipitation and crystallization methods under conditions not exceeding pH 6.8 and not exposing the toxin to any synthetic organic solvents, resins, or protein substances that might be carried over in trace amounts into the final crystalline toxin (3,4)
3. A maximum ultraviolet (UV) absorbance at 278 nm of the toxin dissolved in sodium phosphate buffer at pH 6.8
4. A 260/278 nm absorption ratio of 0.6 or less
5. A specific toxicity of 3×10^7 mouse $LD_{50} \pm 20\%$ per milligram of protein
6. A characteristic banding pattern on gel electrophoresis

To obtain 200 mg of recrystallized toxin for the human trials it was necessary to culture several 16-L batches of type A *C. botulinum*, combine the acid precipitates from the spent cultures, and purify, crystallize and recrystallize the toxin to make up batch 79-11. One hundred mg of this batch (79-11) was sent to Scott's laboratory in San Francisco, and the remainder was retained in Schantz's laboratory at the Food Research Institute as a backup supply and for further research. This batch supplied all of the toxin for the volunteer trials, and on the basis of the quality of the toxin and the success attained in human treatment, it was recommended by Dr. Carl Lamanna of the FDA in 1982 that it be considered as an orphan drug. The Orphan Drug Law was enacted in 1983. The toxin was licensed by the FDA in December 1989 as an orphan drug for the treatment of strabismus, hemifacial spasm, and blepharospasm. It has also come into use for treatment of many other dystonias including spasmodic torticollis, spasmodic dysphonia, writer's and musician's cramps, and similar involuntary hyperactive muscle disorders, as described in various chapters of this book.

The production of toxin in workable quantities in the simplified medium as specified above required cultures of at least 12 or 16 L. Cultures of this size require a step-up inoculum for rapid growth and good toxin production. For this purpose we use 500 ml of the simplified medium at pH 7.3–7.4 inoculated with type A Hall strain of *C. botulinum* and incubated at 37°C. When in the log phase of growth (about 16 hours) it was used as the inoculum for the large cultures. The 12- to 16-L cultures incubated at 37°C attained their maximum growth in 16 to 20 hr, and lysis of the cells began shortly after and was complete in 48 to 72 hr. An estimate of the toxin produced in the cultures was made from the mouse assay using the factor of 3×10^7 mouse LD_{50} (U*) equivalent to 1 mg of toxin per milliliter. A 16-L spent culture at an assay of 10^6 U/ml contains 530 mg of toxin.

The purification of the toxin for human treatment was carried out by simple precipitation and crystallization procedures to avoid contamination, as indicated in the specifications. The type A toxin in the carboys was precipitated by adjustment of the pH to 3.5 with acid. After the precipitate was washed with water and the toxin extracted with 1 M salt solution at pH 6.5–6.8, it was reprecipitated at pH 3.7. To remove much of the nucleic acid and some other impurities, the toxin was extracted from this precipitate with 0.05 M phosphate buffer at pH 6.8 and reprecipitated with 15% grain alcohol at −5°C. For crystallization the toxin was dissolved in sodium phosphate buffer at pH 6.8 and allowed to crystallize after addition of 0.9 M ammonium sulfate solution over a week or two at 4°C. All toxin for human treatment was recrystallized and stored in the mother liquor at 4°C or frozen.

* We have used the capital letter U to designate one mouse LD_{50} or 0.033 ng of purified toxin.

The amount of crystalline toxin expressed in milligrams is obtained from the absorbance at 278 nm using the extinction coefficient of 1.65 per milligram of toxin per milliliter in a 1-cm light path. In this manner of calculation the amount of crystalline toxin from 16 L of spent culture is 85 mg, about a 16% yield, and after recrystallization 42 mg, or an 8% yield.

Purity of the crystalline toxin has been based to the largest extent on the 260/278 nm absorbance ratio, which indicates the relation of nucleic acid impurities to toxin. The ratio indicates the presence of nucleic acids, which have a large solution absorptivity at 260 nm. A ratio of 0.6 or less indicates that the amount of nucleic acid in a crystalline preparation is less than 0.1%. Nucleic acid was removed by careful ethanol precipitation and crystallization rather than by adding RNases as is done in some procedures. Analytical gel electrophoresis has been used to compare independently produced batches, and good similarity has been observed. Only slight differences have been observed, and minor bands have been detected in older batches of toxin. The significance of these observations is not known but is under investigation.

The crystalline toxin stored as a suspension in the mother liquor (0.05 M sodium phosphate buffer at pH 6.8 and 0.9 M ammonium sulfate) of the second crystallization is very stable at 4°C or less for many years and constitutes a reserve supply of toxin (3). For dispensing and treatment by Scott's method,* portions of the crystals were drawn from the bulk supply, dissolved in 0.85% sodium chloride solution containing 0.5% human serum albumin (HSA), and diluted to a workable concentration for injection, usually 100 ng/ml. The purpose of the HSA is to stabilize the toxin on such great dilution. One-tenth milliliter of this solution or 10 ng was placed in small serum bottles and dried by lyophilization. However, mouse assays for toxicity in this medium showed a loss of 80–90% in our laboratory, and Scott reported a similar result. To compensate for this loss it was necessary for Scott to increase the concentration of toxin to 25 ng per vial to recover 100 U. The quantity of toxin required to obtain 100 U in this procedure varies for different batches of toxin. Scott's procedure also included filtration for sterility before drying. Subjecting toxin to filtration in our laboratory showed no loss of toxicity, indicating that loss occurs during formulation and drying. The dried formulation when dissolved in 1 ml of water approximated that of body fluids having a pH of 7.3 and a sodium chloride concentration of 85%. Other formulations were investigated at our laboratory and full recovery of toxicity was obtained in solutions at pH 6.2 and 6.8 containing HSA or bovine serum albumin (8). Although this formulation diverges from the composition of body fluids, it may be preferred to prevent loss of toxicity and possible formation of a toxoid.

Stability of the toxin is very important for reliability in dispensing and dosage. Because of the poor stability of the isolated neurotoxin separated from the protective nontoxic proteins, it was decided by Scott and Schantz that only the crystalline toxin should be used in compounding for medical use. Pure neurotoxin can be kept for several weeks in the cold (4°C) but is more prone to inactivation on dilution, formulation, and drying.

The crystalline type A toxin is a unique compound protein with a molecular weight of 900,000 Mr, composed of only natural amino acids (9), that possesses one or more neurotoxic molecules of 150,000 Mr noncovalently bound to several nontoxic protein molecules that play an important role in the stabilization of the neurotoxic properties. The structure of the toxin and other properties are given in other chapters of this volume.

Type A toxin is soluble in dilute aqueous salt solutions at pH 4–6.8. At pH above 7 the

*The method used by Allergan to produce Botox® is proprietary and may differ.

stabilizing nontoxic proteins dissociate from the neurotoxin, resulting in gradual loss of toxicity, particularly as the pH and temperature rise. Although the crystalline toxin is most stable below pH 7, it can be readily destroyed in solution when heated above 40°C. Toxin in foods is completely destroyed when heated to boiling and even when heated at 80°C for 1 min (10). In general, the best conditions for stability of the toxin in solution are pH 4.2–6.8 and at temperature below 20°C. It can be frozen and stored for months or years in phosphate buffers at pH 6.2–6.8 or in citrate buffers at pH 5.5. It is very stable for periods of years in acetate buffer at pH 4.2 even at 20°C but cannot be frozen, as this causes complete detoxification, and acetate buffer is not practical for use in many cases. The crystalline toxin is easily inactivated in solution by shaking, which produces bubbles that cause surface denaturation. Loss of toxicity readily occurs in dilutions of high-magnitude such as those of a million or more, which are necessary for human treatment. These were important considerations employed for the storage of the toxin and necessary for the development of a formulation for dispensing and use by physicians.

USE OF TYPES OF BOTULINUM TOXIN OTHER THAN TYPE A

The seven known types of BTX (A through G) that cause paralysis by blockage of acetylcholine at the myoneural junction have been isolated and characterized (11). Although type A is the only type licensed by the FDA for treatment of dystonias, it is proposed that types other than A will be used clinically, particularly in patients who develop antibodies or become refractory to type A (12–15). Use of types B and F for human treatment has already been investigated (16,17). Types A, B, and E have been most commonly involved in human botulism (4,11) and type F has been implicated in at least two outbreaks of food poisoning (18). Toxins produced by nonproteolytic strains of *C. botulinum* types B, E, and F must be treated with a proteolytic enzyme to activate them or change the structure of a poorly toxic molecule to one of full toxicity (11). Treatment of type E toxin in spent culture increases the toxicity of the molecule over 100-fold. Toxins produced by proteolytic strains of *C. botulinum* types A, B, and F are activated in spent culture by proteolytic enzymes produced in the culture. It has not been confirmed that types C1, D, and G cause human botulism, but they may act similarly to type A on injection into humans. The different toxin types are differentiated by their distinctive serological specificities, but some cross-reaction can occur. Types E and F show some cross-reactivity (19), and C1 and D can also cross-react (20). Evidence is accumulating that the different types bind to different receptors and have slightly different modes of action (21–23), and could complement type A in clinical applications.

All toxin types in culture fluids consist of the neurotoxin associated with nontoxic proteins that contribute to stability of the neurotoxin. All of the neurotoxins except type G have been isolated, and the fully activated neurotoxins all have a specific toxicity ranging from to 1×10^7 to slightly more than 10^8 mouse LD_{50}/mg protein (11). The complete amino acid sequences for types A through E have been established (24–29). The toxins may act somewhat differently in humans, and in human food-borne botulism serotypes A, B, and E show some differences in severity. Less severe forms of botulism, in which the course of the illness is milder, have also been reported, particularly for type B (30). Differences in severity have also been observed in infant botulism, in which type A causes many of the severe cases requiring the longest hospital treatments (31). These differences in food-borne botulism might be reflected in the value of the toxin types for human treatment.

SAFETY CONSIDERATIONS FOR WORKING WITH *CLOSTRIDIUM BOTULINUM* AND ITS NEUROTOXINS

Botulinum toxin is currently produced as a sterile, lyophilized preparation of crystalline BTX-A. Each vial prepared in the United States contains 100 U (±30 U) of toxin with 0.5 mg human serum albumin and 0.9 mg sodium chloride as bulking agents. The product is reconstituted with 0.9% sodium chloride injection and stored under refrigeration. As labeled, the reconstituted product should be used within 4 hours.

Botulinum toxin is used clinically by injection of 1 to 300 mouse units. Physicians who work with these relatively low quantities of toxin probably do not require immunization with botulinum toxoid. For safety reasons, personnel who work with relatively large quantities of toxin should be immunized by a schedule of injections of pentavalent toxoid available from the Centers for Disease Control (CDC), Atlanta, Georgia. Botulinum toxin is not flammable or volatile; however, mists or aerosols can form that can result in dangerous exposure. Spills can be inactivated by exposure to 0.5% sodium hypochlorite, and solutions of toxin are readily destroyed by heating to boiling or by autoclaving at 121°C for 20 min.

The minimum toxin quantity to cause human food poisoning is not known but has been estimated to be about 3500 mouse intraperitoneal doses of type B toxin (32). The dose necessary to cause severe botulism by food poisoning in humans was estimated to be 0.1 to 1 μg (3000 to 30,000 mouse LD_{50}) (33). Since the toxin causing food poisoning is ingested, the dose necessary for intoxication by injection may be lower. On the basis of studies with adult primates, the lethal dose on injection in humans has been estimated as approximately 1 ng (30–40 U)/kg of body weight (34).

ASSAY PROCEDURES FOR BIOLOGICAL STANDARDIZATION AND CONTROL

The determination of the paralyzing activity (toxicity) of BTX is most important for proper dispensing and dosage. The toxic and immunological properties of the BTX molecule are separate and distinct entities, and analytical methods based on both are in use in some cases. The usual quantal test (35) with mice measures only the toxic or paralyzing action, which is most important in medical treatment. This test is not type-specific and can be used for any of the seven serological types of toxin. Tests based on the immunological properties of the toxin molecule measure the inactive or detoxified molecules as well as active or toxic molecules for any one type and of course do not give a true measure of the paralyzing action. It is essential therefore that assessment of the medical value of the toxin be based on its paralyzing action in an animal, preferably on the mouse test (35).

Mouse assays for the quantitative determination of BTX activity from various laboratories involved in assays in foods have shown a wide range of results. Twofold differences are common, but differences as high as four- and fivefold have been reported (35). The variation appeared to be due mainly to differences in the strains of mice used, but the conditions under which the mice are housed and variations in the techniques used by the assayers also are important. In an attempt to obtain more accurate and precise results from the mouse assay, a reference standard for BTX-A was developed at the Food Research Institute, and a standardized assay for its use was undertaken by the FDA Division of Microbiology in 1975 (35). The purpose of the reference standard was to provide the

assayer at a particular laboratory with a solution containing a definite amount of toxin that was also supplied to all other laboratories carrying out assays for the toxin. In this way each laboratory can determine the nanograms of toxin equivalent to one mouse LD_{50}, and this factor can be used for comparison with other laboratories. A limit of $\pm 20\%$ for differences between laboratories throughout the world should be established, and this is attainable through the use of a reference standard set down by the FDA, the World Health Organization, or International Association of Biological Standardization (36) similar to the one established by the FDA in 1975 (35).

Because all serotypes of BTX appear to have a similar mode of action in causing botulism by a presynaptic block of the release of acetycholine, it should be allowable to express the concentration of other types as type A equivalents. The justification for such a proposal is that type A toxin is the only type readily available in crystalline form that has been researched to a considerable degree. It is uniform in chemical and physical properties from one preparation to another and uniform in toxicity, with a specific toxicity of $3 \times 10^7 \pm 20\%$ LD_{50}/mg.

Other so-called rapid methods include intraperitoneal injection of 0.5 ml of toxin solution (6) in a mouse or the injection of 0.1 ml into the tail vein of the animal (37). When properly carried out these assays yield results within 1 to 3 hours, but the values obtained have two to three times the variability as those yielded by the quantal assay and are used for estimates only.

DISCUSSION

The use of BTX for human treatment by injection has raised new considerations regarding the methods employed for production and purification. Purity and high toxicity with good stability has been the main concern. Because the toxin is a bacterial product, it is likely that one of the main contaminants would be nucleic acids, and we used the 260/278 nm ratio as a measure of purity of the crystalline toxin. At a ratio of 0.6, we have determined that the amount of nucleic acid absorbing material at 260 nm is less than 0.1%. Recrystallization of the toxin usually brings the ratio well below this value, but on each recrystallization we experience a loss of about 50% of the toxin. We recommend the use of the toxin after the second crystallization until it is known whether there is an advantage in using toxin from repeated crystallizations. A ratio of 0.50 appears to be the lowest attainable, because of absorption of the toxin itself at 260 nm.

Another test to help determine the purity of crystalline type A toxin is analytical gel electrophoresis. In conventional electrophoresis, and also in gel electrophoresis, the type A toxin moves as a single component and batch after batch appears similar (4). On reduction and treatment with sodium dodecyl sulfate, which breaks the disulfide and non-covalent bonds, gel electrophoresis has shown a markedly similar but not identical patterns from one batch to another. These small differences in toxin production by the organism have not been resolved but may be a factor in the 50–90% variations Scott observed during lyophilization of different batches.

At the present time batch 79-11 is about 13 years old and is the only batch approved by the FDA. We have attempted over many years to improve the quality of botulinum toxin, and much has been learned of the biochemical and pharmacological properties of type A toxin that is germane to medical use. Further research, particularly in the area of production, purification, and stabilization of toxin preparations, could result in a better-quality product.

REFERENCES

1. Scott AB. Botulinum toxin injection of eye muscles as an alternative to strabismus surgery. Ophthalmology 1989;87:1044–1049.
2. Scott AB. Botulinum toxin injection of eye muscles to correct strabismus. Trans Am Ophthalmology Soc 1981;79:734–770.
3. Schantz EJ, Scott AB. Use of crystalline type A botulinum toxin in medical research. In: Lewis GE, ed. Biomedical aspects of botulism. New York: Academic Press, 1981:143–150.
4. Schantz EJ, Johnson EA. Properties and use of botulinum toxin and other microbial neurotoxins in medicine. Microbiol Rev 1992;56:80–99.
5. Snipe PT, Sommer H. Studies on botulinus toxin. 3. Acid precipitation of botulinus toxin. J Infect Dis 1928;43:152–160.
6. Lamanna C, Carr CJ. The botulinal, tetanal, and enterostaphylococcal toxins: A review. Clin Pharmacol Ther 1967;8:286–332.
7. Schantz EJ. Purification and characterization of *C. botulinum* toxins. In: Lewis KH, Cassel K Jr, eds. Botulism. Proceedings of a symposium. Cincinnati: Public Health Service, 1964: 91–104.
8. Goodnough MC, Johnson EA. Stabilization of botulinum toxin type A during lyophilization. Appl Environ Microbiol 1992;58: 3426-3428.
9. Buehler HJ, Schantz EJ, Lamanna C. The elemental and amino composition of *Clostridium botulinum* A toxin. J Biol Chem 1947;169:295–302.
10. Woodburn M, Somers E, Rodriguez J, Schantz EJ. Heat inactivation of botulinum toxins A, B, E, and F in some foods and buffers. J Food Sci 1979;44:1658–1661.
11. Sugiyama H. *Clostridium botulinum* neurotoxin. Microbiol Rev 1980;44:419–448.
12. Scott AB. Clostridial toxins as therapeutic agents. In: Simpson LL, ed. Botulinum neurotoxin and tetanus toxin. San Diego: Academic Press, 1989:399–412.
13. Hambleton P, Cohen HE, Palmer BJ, Melling J. Antitoxins and botulinum toxin treatment. Br Med J 1992;304:959–960.
14. Jankovic J, Brin MF. Therapeutic uses of botulinum toxin. N Engl J Med 1991;324: 1186–1193.
15. Jankovic J, Schwartz KS. Clinical correlates of response to botulinum toxin injections. Arch Neurol 1991;48:1253–1256.
16. Ludlow CL, Hallett J, Rhew K, et al. Therapeutic use of type F botulinum toxin. N Engl J Med 1992;326:349–350.
17. Borodic G, Ferrante R, Smith K. Botulinum toxin type B as an alternative to botulinum A toxin: a histologic study. Ophthalmol Plast Reconstr Surg (in press).
18. Green J, Spear H, Brinson RR. Human botulism (type F)—a rare type. Am J Med 1983; 75:893–895.
19. Yang KH, Sugiyama H. Purification and properties of *Clostridium botulinum* type F toxin. Appl Microbiol 1975;29:598–603.
20. Oguma K, Syuto B, Kubo S, Iida H. Analysis of antigenic structure of *Clostridium botulinum* type C1 and D toxins by monoclonal antibodies. In: Macario AJL, de Macario EC, eds. Monoclonal antibodies against bacteria. Vol. 2. New York: Academic Press, 1985:159–189.
21. Gansel M, Penner R, Dreyer F. Distinct sites of action of clostridial neurotoxins revealed by double-poisoning of mouse motor terminals. Pflugers Arch–Eur J Physiol 1987;409:404–416.
22. Kozaki S. Interaction of botulinum type A, B, and E derivative toxins with synaptosomes of rat brain. Naunyn-Schniedbergs Arch Pharmacol 1979;308:67–70.
23. DasGupta BR. Structure and biological activity of botulinum neurotoxin. J Physiol (Paris) 1990:84:220–228.
24. Binz T, Kurazono H, Wille M, Frevert J, Wernars K, Niemann H. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J Biol Chem 1990;265:9153–9158.

25. Thompson DE, Brehm JK, Oultram JD, Swinfield, Shone CC, Atkinson T, Melling J, Minton NP. The complete amino acid sequence of the *Clostridium botulinum* type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene. Eur J Biochem 1990;189: 73–81.
26. Whelan SM, Elmore MJ, Bodsworth NJ, Brehm JK, Atkinson T, Minton NP. Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of the entire nucleotide sequence. Appl Environ Microbiol 1992;58:2345–2354.
27. Hauser D, Eklund MW, Kurazono H, Binz T, Niemann H, Gill DM, Boquet P, Popoff MR. Nucleotide sequence of the *Clostridium botulinum* C1 neurotoxin. Nucl Acids Res 1990;18:4924.
28. Binz T, Jurazono H, Popoff MR, Eklund MW, Sakaguchi G, Kozaki S, Krieglstein K, Henschen A, Gill DM, Niemann H. Nucleotide sequence of the gene encoding *Clostridium botulinum* neurotoxin type D. Nucl Acids Res 1990;18:5556.
29. Whelan SM, Elmore MJ, Bodsworth NJ, Atkinson T, Minton NP. The complete amino acid sequence of the *Clostridium botulinum* type E neurotoxin, deduced by nucleotide sequence analysis of the encoding gene. Eur J Biochem 1992;204:657–667.
30. Cruz-Martinez A, Anciones B, Ferrer MT, Tejedor D, Prez Conde MC, Bescansa E. Electrophysiologic study in benign botulism. Muscle Nerve 1985;6:448–452.
31. Arnon SS. Infant botulism. In: Feigin RD, Cherry JD, eds. Textbook of pediatric infectious diseases. 3d ed. Philadelphia: W.B. Saunders, 1992:1095–1102.
32. Meyer KF, Eddie B. Perspectives concerning botulism. Z Hyg Infektionskr 1951;133: 255–263.
33. Schantz EJ, Sugiyama H. Toxic proteins produced by *Clostridium botulinum*. J Agr Food Chem 1974;22:26–30.
34. Gill DM. Bacterial toxins: a table of lethal amounts. Microbiol Rev 1982;46:86–94.
35. Schantz EJ, Kautter DA. Standardized assay for *Clostridium botulinum* toxins. J Assoc Off Anal Chem 1978;61:96–99.
36. Cockburn WC, Hobson B, Lightbown JW, Lyng J, Magrath D. The international contribution to the standardization of biological substances. II. Biological standards and the World Health Organization 1947–1990. General Considerations. Biologicals 1991;19:257–264.
37. Boroff DA, Fleck U. Statistical analysis of a rapid in vivo method for the titration of the toxin of *Clostridium botulinum*. J Bacteriol 1966;92:1580–1581.